HERRING, ASHLEY D., M.S. Localization of Myosin II and Identification of Its Potential Upstream Regulators in *Dictyostelium discoideum*. (2015)
Directed by Dr. Paul A. Steimle. 49 pp.

The thesis work presented here aimed to understand how myosin II is regulated in *Dictyostelium discoideum* within the context of chemotactic signaling and cell migration. Myosin II filaments are regulated via phosphorylation of the heavy chain tails, mediated by myosin heavy chain kinases (MHCKs). The MHCKs are a key point of regulation of myosin II filament disassembly. However, little is known about upstream regulators of myosin II and therefore, is a major component of this project. The GFP-tagged version of myosin II was electroporated into cells lacking putative upstream regulators in order to study cAMP-mediated myosin II translocation. To determine if the lack of one of these regulators indeed affected myosin II translocation, the phenotype of the mutants were compared to wildtype Ax2 cells. Cells lacking GbpC and GskA demonstrated loss of the wildtype phenotype, while PkbA null, RegA null, and VwkA null cells showed defects, but not the loss of the wildtype phenotype, indicating that GbpC and GskA are necessary for proper myosin II translocation. Another aim focused on studying the effect of the loss of all MHCKs on myosin II translocation. The MHCK D knockout plasmid (MHCKD-KO) was electroporated into MHCK A/B/C null cells, however, none of the isolates contained the disrupted version of the *mhkD* gene as confirmed by diagnostic PCR. The results indicate that the lack of all MHCKs may be lethal to the cells and require a knockdown approach instead. In conclusion, myosin II filament regulation is highly coordinated and relies on a variety of upstream regulators, the loss of which inhibits myosin II function. In addition, the loss of all MHCKs seem to be either be lethal to

Dictyostelium cells, indicating another method is needed to study this effect on myosin II filament disassembly and regulation. These studies offer a basis of future research directed at understanding and pinpointing upstream regulators of myosin II filament regulation within the context of chemotaxis and cell migration.

LOCALIZATION OF MYOSIN II AND IDENTIFICATION OF ITS POTENTIAL UPSTREAM REGULATORS IN *DICTYOSTELIUM DISCOIDEUM*

by

Ashley D. Herring

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2015

Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

Committee Chair_____

Committee Members_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Dictyostelium* – *The Organism

Dictyostelium discoideum is a social amoeba that lives in the soil and sustains itself on the surrounding bacteria. Its life cycle can be characterized by a growth phase and a multicellular developmental phase (Figure 1). During the growth phase, *Dictyostelium* exists in a unicellular amoeboid state, feeding on bacteria until the food source is depleted. Once the environment is depleted of nutrients, the amoebae shift into a developmental phase. At this point, starvation induces down-regulation of growth genes and triggers up-regulation of developmental genes. A progenitor cell initially releases pulses of cyclic adenosine monophosphate (cAMP) pulse, which binds to receptors on the surfaces of cells. In turn, these cells are stimulated to release more cAMP, propagating the signal outward and extending the concentration gradient further out, ultimately leading to the recruitment of more cells to the aggregation center. Eventually, the cells attracted by the cAMP will form a loose aggregate which then becomes more defined, forming a mound with a tip. The tip will drive elongation of the mound of cells which now contains cells that have undergone predifferentiation and are targeted to eventually become either stalk or spore cells, to form either a standing slug (finger) or a pseudoplasmodium (migrating slug). The migrating slug is both thermotaxic

and phototaxic, which aids in its relocation to sites where there is a greater availability of nutrients. If nutrients are still not available after migration, the slug will stop and continue with the developmental process that ultimately leads to the formation of a fruiting body structure comprised of a thin stalk composed of dead cells and a cap that contains spores of *Dictyostelium* cells. When the fruiting body is disturbed, spores are released, and if conditions are conducive, the spores will germinate, releasing single cells that feed on nutrients in the area (Kessin, 2001).

Dictyostelium is a readily accessible, relatively simple, and well-studied organism, especially in regard to cellular processes common to higher eukaryotes. *Dictyostelium* studies have revealed that the organism is a valuable model for studying the molecular events driving and regulating basic cellular processes such as cell migration, multicellular development, and cytokinesis (Sasaki et al., 2002). Several biochemical, cellular, and genetic studies in *Dictyostelium* have shown that myosin II is essential for these processes. Therefore, myosin II would be a key protein to investigate in order to understand the regulation of the previously mentioned processes.

***Dictyostelium* Myosin II**

The *Dictyostelium* genome contains a number of different types of myosins, all of which share homologous head groups at the amino terminal end of the myosin heavy chain that mediate the contraction of actin filaments. However, these myosins differ from each other in the structural and functional properties of the C-terminal regions of their heavy chains. In particular, only myosin II ("conventional" myosin) possesses a C-

terminal region that mediates the formation of myosin II bipolar filaments, conferring the ability of myosin II to contraction actin filaments. *Dictyostelium* possesses only one copy of the gene for conventional, double-headed myosin II. Myosin II is a hexameric protein consisting of two regulatory chains, two essential light chains, and two essential heavy chains (Figure 2). The heavy chains contain N-terminal motor domains, which are necessary for the ATP-dependent contraction of actin filaments (Bosgraaf and van Haastert, 2006). However, myosin II must exist as bipolar filaments in order to do so.

Myosin II bipolar filaments are formed via the regulated interaction of the coiled-coil domains of the MHC tails of individual myosin II hexamers. As filaments, myosin II interacts with the actin cytoskeleton to drive cell shape change and provide general stability to the cell cortex (Xu et al., 2001). During chemotaxis, this myosin-actin cytoskeletal complex becomes enriched at the cell rear and is central to establishing the polarity of the cell and the directionality of its movement. In addition, the cytoskeletal complex will localize to the cleavage furrow to assist in cytokinesis, incorporating the ATP-dependent activity of myosin II (Bosgraaf and van Haastert, 2006). Since myosin II filaments play an integral part in important cell processes, its activity is highly regulated. Myosin II bipolar filament assembly is regulated through phosphorylation of the heavy chain (MHC) tails (Yumura et al., 2005). Past mutagenesis studies revealed phosphorylation sites on the MHC tails to be three threonines at positions 1823, 1833, and 2029 (Vaillancourt et al., 1988). Additionally, when these threonines were changed to nonphosphorylatable alanines (3XALA), *D. discoideum* cells exhibited significant myosin II overassembly within the cytoskeleton (Egelhoff et al., 1993). Furthermore,

3XALA cells exhibit major defects in chemotaxis, including decreased polarity and increased development of improperly positioned pseudopodia (Stites et al., 1998). The cells were unable to recover the normal myosin II phenotype, suggesting that myosin II must cycle between phosphorylated and dephosphorylated states in order to function correctly.

Myosin II contractile activity, and thus filament assembly, is absolutely required for normal cytokinesis. Previous research has uncovered that myosin II localizes to the cleavage furrow of cells, and is critical for proper cytokinesis when grown in suspension culture (Manstein et al., 1989). However, myosin II is not necessary for cytokinesis to occur on an adherent surface. Nevertheless, even when attached to a surface, cells containing a heavy chain null version of myosin II still exhibit delays in furrow localization (Weber et al., 2000), and resulting daughter cells exhibit defects in the amount of proteins, organelles, and other cytoplasmic materials present within each cell (Zang et al., 1997). This suggests that myosin II may be regulated by a variety of upstream regulators, which are dependent on where and when myosin II will be localized to a particular region of the cell.

Myosin II Heavy Chain Kinases

A crucial part in understanding the role of myosin II in driving cell shape change lies in knowing the main upstream regulators of this motor protein. One main class of regulators of myosin II are myosin heavy chain kinases (MHCKs). *Dictyostelium discoideum* have an array of MHCKs that drive myosin II filament assembly both *in vitro*

and *in vivo* (Figure 3). Previous gene disruption studies of MHCK A, B, and C also add support roles for these kinases in myosin II filament regulation (Kolman et al., 1996; Rico and Egelhoff, 2003; Nagaski et al., 2002).

Molecular and biochemical analyses of the *Dictyostelium* MHCKs have revealed that these kinases contain WD-repeat domains at the carboxyl-terminal ends and centrally located alpha kinase catalytic domains (Figure 4). The name "alpha kinase" arose from early studies identifying a preference for this family of enzymes to phosphorylate alpha helical regions of proteins. A fourth heavy chain kinase, MHCK D, also has a carboxyl-terminal WD-repeat domain and an alpha kinase catalytic domain. Because protein structure usually corresponds with its function, MHCK D may regulate myosin II assembly in a similar fashion as the other three MHCKs. However, limited research has been completed on the role of MHCK D. Therefore, my thesis research, in part, investigated the role of MHCK D, specifically in the regulation of myosin II, by attempting to generate a quadruple knockout cell line in which the *mhkD* gene has been disrupted in *Dictyostelium* cells already lacking expression of MHCK A, B, and C. Previous research investigated the differences in morphology and biochemical properties between wild type and *mhkA/mhkB/mhkC* null *Dictyostelium* cells (Nagasaki, 2002). Consequently, by generating an *mhkA/mhkB/mhkC/mhkD* null cell line, I can compare noted differences between this cell line and *mhkA/mhkB/mhkC* cells.

Potential Upstream Regulators of Myosin II Localization

In addition to the MHCKs, other *Dictyostelium* proteins appear to play roles in regulating myosin II filament assembly (Figure 5). For example, one study demonstrated that disruption of another alpha kinase, von Willebrand factor kinase A (VwkA), inhibited cytokinesis of *Dictyostelium* when cells were grown in suspension culture (Betapudi et al., 2005). Additionally, VwkA shares some homology with MHCKs, suggesting a significant role in regulating myosin II filament assembly. Another study found that when RegA, a cAMP-specific phosphodiesterase, was disrupted, cells expressed more lateral pseudopodia and had decreased chemotactic efficiency (Wessels et al., 2000), suggesting additional functions besides phosphodiesterase activity. Furthermore, protein kinase B (PKB) localizes to the plasma membrane when stimulated with cAMP. When the gene (*pkbA*) was disrupted, cells exhibited defects in aggregation (Meili et al., 1999). In addition, cells lacking cyclic guanine monophosphate (cGMP) binding protein (GbpC) also show defects in chemotaxis and MHC phosphorylation when stimulated with cAMP (Bosgraaf et al., 2002). Finally, mutagenesis studies show that glycogen synthase kinase 3, encoded by *gskA*, is required for efficient chemotaxis (Teo et al., 2010), suggesting that this gene plays a role in regulating myosin II function as well. Therefore, studying cells that lack one of these genes may provide insight into how myosin II filaments are regulated.

As previously stated, myosin II plays a significant role in proper chemotaxis and cytokinesis. The above mentioned potential upstream regulators are suspected to play a role in efficient myosin II function. Therefore, disruption of the corresponding genes

may inhibit proper myosin II function, thus making these regulators important in understanding proper myosin II localization and function in the cell. My research examined the effects of disrupting the expression of these putative regulators on cortical myosin II filament assembly in *Dictyostelium* cells.

Previous experiments with fluorescence microscopy have allowed researchers to visualize proteins within live cells. With this approach I can visualize myosin II localization within various cell lines. Given that these upstream regulators are suspected to regulate myosin II filament assembly, I hypothesize that cells lacking these crucial upstream regulators of myosin II will show defects in myosin II assembly. In addition, cells require all four MHCKs for proper chemotaxis and cytokinesis, and therefore, when disrupted, cells will exhibit severe defects in myosin II assembly. Together, my thesis research seeks to understand how myosin II is regulated, especially in the context of cell migration and chemotaxis in order to gain insight on how these processes would be regulated in higher eukaryotes.

Thesis Research Aims

The overarching goal of this thesis project is to understand how myosin II is regulated in *Dictyostelium discoideum* within the context chemotactic signaling and cell migration. To this end, my thesis research involved studies to analyze the effects of removing putative and known upstream regulators of myosin II on the ability of myosin II to undergo chemoattractant-stimulated translocation to the cell cortex. Previous studies of *Dictyostelium* cells (as well as those of other organisms) have established that myosin II localizes to the rear of chemotaxing cells where it plays a critical role in facilitating

detachment of the cell from the substrate and movement of the cell body forward. In order for myosin II to be recruited to a specific region of the cell cortex (e.g. cell rear), it must first assemble into bipolar filaments.

In *Dictyostelium*, myosin II filament turnover is regulated directly via the coordinated activities of four myosin heavy chain kinases (MHCKs A, B, C, and D); however, at this time there is little known about the roles of upstream signaling components in regulating myosin II filament assembly/disassembly in response to cAMP stimulation of cells. Moreover, our understanding of how the MHCK family of enzymes collectively regulates myosin II is still incomplete, since it is not known how myosin II localization and activity is affected in cells lacking all four of the known MHCKs in *Dictyostelium*. As a means of gaining insight into the mechanisms by which myosin II filament assembly and localization is regulated in response to chemoattractant stimulation, my thesis project involved pursuing the following two aims:

1. **Determine myosin II localization in cells lacking putative upstream regulators of myosin II filament assembly:** Numerous studies have established that myosin II undergoes a rapid change from a primarily cytoplasmic distribution to a cortical localization in response to stimulation of cells with a uniform and saturating concentration of cAMP. For the first aim of my thesis research project, I examined cAMP-stimulated myosin II relocalizaiton in a set of cell lines lacking expression of putative upstream regulators of chemoattractant-stimulated assembly and cortical recruitment of myosin II (Table 1). If one of these genes indeed regulates myosin II filament turnover and localization, then cells lacking expression of that gene are

predicted to exhibit altered myosin II localization properties (e.g. myosin II remains cytoplasmically localized after cAMP stimulation of cells).

Cell lines have been obtained in which each of the candidate genes has been knocked out. Each cell line was transformed with recombinant plasmid for the expression of GFP-tagged myosin II, and expression of the fusion protein was confirmed visually (via fluorescence microscopy) and immunoblot analysis of cell lysates using anti-GFP antibody. Cell lines with confirmed expression of GFP-myosin II were then prepared for cAMP stimulation by placing them in starvation conditions for 8 to 12 hours, after which cells were placed on a coverslip and allowed to adhere to the glass surface. Cells were examined for chemoattractant-stimulated translocation of GFP-myosin II by treating the cells with cAMP, then collecting images every 10 seconds (up to 120 seconds). GFP-myosin II localization in mutant cells lines were compared to wild type (AX2) cells (positive control).

2. **Examine myosin II localization dynamics in cells lacking all four MHCKs:**

As a means of gaining a more complete understanding of the roles myosin heavy chain kinases play in regulating myosin II filament assembly and cellular localization I attempted to generate a cell line in which all four of the known *Dictyostelium* MHCK genes (*mhkA*, *B*, *C*, and *D*) have been disrupted. To this end, triple knockout *Dictyostelium* cells (lacking MHCKs A, B, and C) were transformed with a plasmid (pBsr-Nsi-MHCKD-KO) that has been used previously to create a single knockout cell line in which the *mhkD* gene has been disrupted via homologous recombination. A particular challenge with this strategy is that the selectable marker associated with

the *mhkD* knockout plasmid (blasticidin resistance) was already introduced with the generation of the 3x KO cell line that was used. To overcome this problem, I screened for 4x KO cells by isolating genomic DNA from clones of cells transformed with the pBsr-Nsi-MHCKD-KO plasmid, followed by PCR analysis to identify transformants where the *mhkD* gene has been replaced with the disruption cassette. Positive clones were then to be transformed with a GFP-MYO plasmid for expression of GFP-tagged myosin II in order to study changes in cAMP-stimulated recruitment of myosin II, as described in the first aim of this proposal. Based on the known roles of the four MHCKs in regulating myosin II bipolar filament turnover, it is predicted that the quadruple knockout cells will exhibit detectably increased levels of cortically localized myosin II. This was to be explored by examining the newly generated *Dictyostelium* 4x KO cells for their ability to undergo normal cytokinesis and development, both of these processes require highly regulated changes in myosin II filament assembly and localization in the cell.

CHAPTER II

MATERIALS AND METHODS

Maintenance of Dictyostelium Cell Lines

Cell lines used in this work were obtained from DictyBase Stock Center (Table 2). All *Dictyostelium* cell lines were cultured axenically in 10cm Petri dishes with HL5 medium containing penicillin and streptomycin (Sussman, 1987). The HL5 medium (pH 6.5) was made using dextrose (Fisher), yeast extract (Oxoid), proteose peptone (Oxoid), potassium phosphate (Fisher), and sodium phosphate (Fisher). Cells containing the GFP-tagged myosin II (GFP-MYO) plasmid have a geneticin (G418) antibiotic resistance gene, and the medium used to grow the cells was supplemented with 10 μg/ml of G418 (CalBioChem) for selection.

Cell Electroporation

This protocol was used to introduce the GFP-MYO and MHCKD-KO plasmids into *Dictyostelium* cells. Plasmids were introduced into all cell lines using the following method: Confluent cells were collected from plates and counted using a hemocytometer. The volume needed for 1.5×10^7 cells was then transferred to a Falcon tube and centrifuged for 5 minutes at 2,500 rpm. The supernatant was discarded, and the pellet washed with 1 mL of H50 buffer solution (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1mM $MgSO_4$, 5 mM $NaHCO_3$, and 1 mM NaH_2PO_4) and transferred to a 1.5 mL micro-

centrifuge tube. The cells were centrifuged again at the before mentioned settings. This wash and centrifugation was repeated once more. Afterwards, the pellet was then resuspended in 300 µl of H50 buffer. From this, 100 µL was added to Bio-Rad Gene Pulser cuvettes (0.1 cm space between plates). The plasmid was added to one cuvette of cells, while the other cuvette contained only cells as a negative control. A current was applied to each cuvette (0.85 mV), then placed on ice. After 5 minutes, the cells were transferred to sterile 10 cm Petri dishes with HL5 medium, and the selecting agent (G418) was added to the GFP-MYO plasmid cells the following day.

Cells electroporated with the MHCKD-KO plasmid were plated at a low density using the following dilution series: 900 µL of HL5 medium was added to the cuvette (containing the 100 µL of cells) to bring the total volume to 1mL. This volume was added to a Falcon tube containing 9 mL of HL5 medium, then inverted to mix the cells. From this tube, 500 µL was transferred to another Falcon tube with 4.5 mL of medium. This step was repeated 3 more times to bring the final dilution to 1/100,000. The final tube (5 mL) was transferred to a new 10 cm Petri dish with 3 mL of HL5, and cells were allowed to grow until colonies formed.

***Dictyostelium* Cell Line Storage**

To ensure that cells would be available for continuing and future studies, all cell lines were either stored in either 10% glycerol or 20% dimethyl sulfoxide (DMSO) aliquots. Cell lines stored in 10% glycerol stocks were prepared in following manner: Once plates reached approximately 90% confluency, cells were collected in 1 mL of starvation buffer (20 mM MES, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, pH 6.8). The volume of

cells were plated on starvation plates (20 mM MES, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, 15g/L of agar, pH 6.8), and set under a ventilation hood for 15-20 minutes to allow the cells to adhere. After spores formed (usually after 24 hours), they were collected using disposable inoculating loops and deposited in 100 µL of 10% glycerol, then placed in a -80°C freezer.

Spores were saved in 20% DMSO were prepared as follows: Once cells reached approximately 90% confluency, the HL5 medium was replaced with 1 mL of Tris-Bis medium (pH 6.8). Following this, 1 mL of Tris-Bis medium (pH 6.8, 20% DMSO) was added to the plate and tipped to mix. The cells were collected using this solution, saved in two 1.5 mL microcentrifuge tubes, and stored at -80°C.

Conventional Fluorescence Microscopy

Chemoattractant-stimulated translocation of GFP-tagged myosin II was visualized via fluorescence microscopy (epifluorescence) of live cells. Confluent cells expressing the GFP-MYO protein were collected into Falcon tubes and counted using a hemocytometer. Caffeine was added to the cells at a concentration of 100 mM and allowed to incubate for 30 minutes in order to inhibit existing phosphodiesterases. Following incubation, approximately 5 x 10^4 cells were transferred to circular glass coverslips (Fisherbrand 12-546-2) and allowed to adhere for 10-15 minutes. The cells were contained by drawing a perimeter with a hydrophobic pen (Pap Pen). Afterwards, starvation buffer was added to the coverslip to bring the final volume to 100 µL. Cells were observed for fluorescence using an Olympus IX70 microscope and images collected every 10 seconds. After 30 seconds, 10 µL of cAMP (1 mM) was added to the drop of

cells (final concentration of 100 μM) and images were collected every 10 seconds up to 120 seconds total. Images were processed using Image Pro-Plus software.

Cell Lysate Preparation

To prepare cells for Western Blot Analysis, cells were lysed using the following procedure: Cells were grown until confluent and counted using a hemocytometer. The volume containing approximately 10^7 cells was transferred to a 15 ml Falcon tube and centrifuged at 2,500 rpm for 5 minutes. Following centrifugation, the supernatant was discarded, and the pellet washed three times with 1 mL of 10 mM Tris pH 7.5. After the final wash, the supernatant was discarded and the pellet placed on ice. Next, the pellet was resuspended in 100 μL of ice cold lysis buffer (50 mM Tris pH 8.0, 20 mM NaPPi, 5 mM EDTA, 5 mM EGTA, 20% Triton X-100) supplemented with PIC (2 mg/mL antipain, 10 KIU/mL aprotinin, 10 mg/mL benzamidine, 1 mg/mL leupeptin, 5 mg/mL Pefabloc, and 10 mg/mL L-1-chloro-3[4-tosylamido]-7-amino-2-heptanone-HCl N-α-tosyl-L-lysine chloromethyl ketone [TLCK] in ddH_2O). Immediately after, 100 μL of preheated (95°C) 4x SDS PAGE sample buffer (50 mM TRIS-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) was added and vortexed for 5 seconds at a medium speed. Immediately following vortexing, the lysate was heated (95°C) for 4 minutes. Lysates were allowed to cool before loading on SDS-PAGE gel, and remaining samples were stored at -20°C.

SDS-PAGE Gel Preparation

SDS-PAGE gels were prepared for Western Blot analysis as follows: 8% Resolving gels were made using 1.6 mL 40% Acrylamide, 2 mL 1.5 M Tris pH 8.8, 80 µL 10% SDS, 80 µL 10% APS and 8 µL TEMED in 4.2 mL dH_2O, for a total volume of 8 mL. The APS and TEMED were added to the mixture last to induced polymerization. Immediately after adding the APS and TEMED, the solution was transferred to a casting tray and allowed to polymerize. Once solidified, the stacking gel (0.75 mL 40% Acrylamide, 1.25 mL 0.5M Tris pH 6.8, 50 µL 10% SDS, 50 µL 10% APS, 5 µL TEMED, 2.9 mL dH_2O) was poured on top with a comb to create wells for the samples.

Western Blot Analysis of GFP-MYO Plasmid Uptake

Cells were analyzed for proper GFP-MYO plasmid uptake and corresponding protein expression using a Western Blot. The following procedure was used: Samples were loaded onto a SDS-PAGE gel and set to 300 mA for 3 hours. After the gel finished running, it was transferred to a cassette and assembled by layering a sponge, 2 pieces of filter paper, the gel, transfer membrane (Immobilon), another 2 pieces of filter paper, and another sponge. The cassette was placed in the transfer chamber with a stir bar in the bottom and an ice pack to keep the chamber cool. The transfer chamber was filled with Western Blot transfer buffer (2.9 g/L glycine, 5.8g/L Tris base, 0.37 g/L SDS, pH 8.3) and allowed to run at 90 mA overnight. The following day, the cassette was removed and the membrane placed in a Ziploc bag with 5 mL of blocking solution (5% BSA, 1% Tween + 1x TBS), then placed on a rotator for 1 hour at 4°C. Following blocking, the membrane was transferred to a new Ziploc bag with 2.5 µL of the primary antibody

(mouse anti-GFP or rabbit anti-myosin heavy chain (MHC)) and 5 mL of fresh blocking solution, and allowed to incubate for 1 hour at room temperature. After incubating, the primary antibody was collected, and the membrane was washed 3 times with fresh TBST for 5 minutes each. Next, the membrane was transferred to a new Ziploc bag with 5mL of fresh blocking solution and 2 μL of the secondary antibody (either goat anti-mouse monoclonal antibody for anti-GFP or goat anti-rabbit polyclonal antibody for anti-MHC), and placed on the rotator for 1 hour at 4°C. After incubation, the secondary antibody was collected, and the membrane was washed with fresh TBST 3 times for 5 minutes each. After the final wash, the membrane was transferred to plastic wrap and 500 μL of Lumi-Phos (Bio-Rad) was distributed evenly over the membrane. After sitting for 4 minutes, the excess Lumi-Phos was discarded. The membrane was imaged using Bio-Rad ChemDoc System and analyzed with the accompanying Quantity One software.

Generation of MHCK $A^-B^-C^-D^-$ Cell Line

MHCK $A^-B^-C^-$ cells were electroporated with the pBsr-Nsi-MHCKD-KO plasmid using the same method stated above. After electroporation, cells were plated at a low density using a 1/100,000 dilution and allowed to grow until colonies formed. Isolates were transferred to separate plates and grown until confluent. Cells containing the pBsr-Nsi-MHCKD-KO plasmid were identified by diagnostic PCR using primers corresponding to the defective cassette version of MHCK D.

PCR Analysis

The PCR analysis was used to confirm proper homologous recombination of the MHCKD-KO plasmid with genomic *mhkD* when establishing the MHCK $A^-B^-C^-D^-$ KO cell line using the method described by Betapudi et al. (2004). Genomic DNA extraction by standard procedures was performed on all cell lines using Qiagen DNeasy Tissue Kit. This DNA (1 µL) was added to a master PCR mix containing 25 µL of GoTaq Green 2x Master Mix (Promega), 5 µL of the forward primer at 10 µM (MHCKD5 – GGTAGAATGCCATCAACTGGC), 5 µL of the reverse primer at 10 µM (MHCKD3 – CCAAACATACGTAATGATGTAATTGG), and 14 µL of nuclease-free water, per reaction tube. All tubes were placed in a PTC-100 PCR thermocycler (MJ Research, Inc.) and cycles were set as follows:

1. Initial denaturation at 94°C for 1 min
2. Denaturaion at 94 for 30 seconds
3. Annealing at 59°C for 30 seconds
4. Extension at 72°C for 1.5 minutes

} Repeated 30 times

After the cycles were complete, 10 µL of the PCR reaction mix was loaded into an agarose gel. The gel was run at 120 V for 45 minutes, then imaged using Bio-Rad ChemDoc System and analyzed with Quantity One software.

CHAPTER III

RESULTS

Western Blot Analysis of GFP-Myosin Plasmid Uptake

To ensure proper uptake of the GFP-tagged myosin plasmid (GFP-MYO) and expression of the full GFP-tagged myosin II protein within each cell line, a Western Blot was performed after electroporation. Because the GFP-tagged myosin II protein can occasionally break down yet still fluoresce, a Western Blot was needed to confirm stable expression of the GFP-MYO protein. The expected size of the intact GFP-MYO protein is approximately 270 kiloDaltons (kDa) due to myosin II being 243 kDa and GFP approximately 25 kDa in size. In Figure 6A, bands appearing at 270 kDa in all lanes show that GFP is most likely still attached to myosin II. Even though the band is smeared in Figure 6B, it is slightly higher than the 250 kDa marker, indicating that the GFP-MYO protein did not break down significantly. Therefore, based on the positions of the bands in the each blot, the full GFP-MYO protein is being expressed in each cell line, with minimal breakdown.

Analysis of Cell Cortex Enrichment Assay

After confirmation of stable GFP-MYO expression via Western Blot, each cell line was assessed for cAMP-stimulated cortical enrichment of myosin II. Prior to cAMP stimulation, cells may or may not have myosin II localized at the cortex. If myosin II is

not at the cortex initially, then when stimulated, myosin II will be recruited to the cell cortex over a period of 30 – 40 seconds, then dissociate to the cytoplasm. If myosin II is localized at the cortex initially, then when stimulated, myosin II will still become further enriched at cell the cortex, followed by dissociation back to the cytoplasm, even though the signal may not be as strong. In contrast, if there are defects in the cell's response to cAMP stimulation, there may either be decreased or no recruitment of myosin II to the cortex. Other defects may be reflected in no dissociation of myosin II from the cortex.

When stimulated with cAMP, wild type Ax2 cells exhibit normal myosin II cortex recruitment (Figure 7). Prior to cAMP stimulation (Figure 7, 0 seconds), there is essentially no localization of myosin II at the cell cortex. At 20 seconds after cAMP stimulation, there is a marked increase in myosin II at the cortex, which remains at 30 seconds after cAMP stimulation. By 60 seconds after cAMP stimulation, the myosin II has dissociated from the cortex and returned to the cytoplasm. Therefore, cAMP stimulated Ax2 cells exhibited the expected myosin II cortex translocation response and were used as the basis of comparison for other cell lines.

While Ax2 cells exhibit proper cAMP-stimulated myosin II translocation, triple alanine (3XALA) cells do not. Cells expressing GFP-tagged myosin II possessing the 3xALA mutation exhibit high levels of cortical myosin II in the unstimulated state, before the addition of cAMP (Figure 8, 0 seconds), there are significant levels of myosin II localized at the cell cortex. Once stimulated, there is an increase in the amount of myosin II recruited to the cortex (Figure 8, 20 seconds). The intensity of myosin II at the cortex remains at 30 seconds post stimulation, and it does not decrease as time continues (Figure

8, 60 seconds). Therefore, when the phosphorylatable threonines are changed to nonphosphorylatable alanines, the 3XALA cells display defects in myosin II filament disassembly when stimulated with cAMP, as has been reported previously.

In contrast to Ax2 cells, GbpC null cells do not exhibit proper translocation of myosin II when stimulated with cAMP. In Figure 9 (0 seconds), myosin II is initially localized to the cortex at the poles of the cell. However, when stimulated with cAMP, myosin II is not recruited to the cortex over the course of 20-30 seconds. Instead, levels of cortical myosin II decrease over this time period, and by 60 seconds, cortical myosin II has decreased to below initial basal levels. This suggests that myosin II recruitment is impaired when *gpbC* is disrupted, and therefore the corresponding protein is necessary for proper myosin II filament assembly.

Likewise, GskA null cells exhibit a similar phenotype as GbpC null cells when stimulated with cAMP. Initially, most of the GFP-MYO protein is localized in the cytoplasm of the cell (Figure 10, 0 seconds). However, when GskA null cells were stimulated with cAMP, they did not display the peak in levels of cortical myosin II seen in wild type Ax2 cells (Figure 7A, 10). Even after 60 seconds post cAMP stimulation, GskA null cells do not exhibit myosin II enrichment of the cell cortex. These results indicate that GskA is essential for proper cAMP-mediated myosin II translocation.

In contrast, PkbA null cells seem to exhibit some cortical localization of myosin II when stimulated with cAMP, even though the pattern of distribution is affected. Prior to cAMP stimulation, there is initially a significant amount of GFP-MYO protein at the cell cortex (Figure 11, 0 seconds), even though it is localized to one particular area.

However, at 20 seconds post cAMP stimulation, there is an increase in the amount of GFP-myosin II at the cortex (Figure 11). After 30 seconds post stimulation, the levels of cortical myosin have decreased, and by 60 seconds, the amount of cortical myosin II levels have returned to their pre-cAMP stimulation levels. These results indicate that PkbA null cells have the ability to complete cAMP-stimulated myosin II translocation. Therefore, even though the cell's distribution of cortical myosin II is affected, PkbA null cells do not exhibit defects in the ability of myosin II to translocate in response to cAMP stimulation.

RegA null cells also display defects in myosin II filament regulation. Originally, GFP-myosin II is localized at the cell cortex (0 seconds, Figure 12). However, once stimulated, the already present cortical myosin II seems to aggregate at one particular spot at the cortex (20 seconds, Figure 12). The levels of cortical myosin II stay relatively constant, simply shifting from an even spread to localized distribution. By 60 seconds post stimulation, the enrichment at said spot in the cell has decreased in intensity. As a result, RegA is not essential for *Dictyostelium* cells to undergo cAMP-mediated myosin II translocation, but is needed for proper localization of myosin II

Lastly, VwkA null cells display an unusual pattern of myosin II localization when stimulated with cAMP. Initially, there are high amounts of GFP-myosin II localized around the cortex towards what appears to be the anterior (leading edge) of the cell (Figure 13, 0 seconds). Once the cell is stimulated, most of the cortical myosin II initially present has dissociated and moved to the cytoplasm (20 seconds). By 30 seconds post stimulation, the GFP-myosin II has not returned to the cortex. However, by 60

seconds after cAMP stimulation, there is a slight recruitment of GFP-myosin II to the cortex. Without VwkA, stimulated cells do not exhibit the rise in myosin II at the cortex over the same time as Ax2 cells. Instead, cortical myosin II levels in VwkA null cells decrease before being recruited to the cortex much later. These results suggest that VwkA may play a role in relaying the cAMP signal to myosin II filaments.

In summary, all experimental lines tested exhibited some form of aberrant myosin II translocation. RegA null, PkbA null, and VwkA null cells seem to show decreased levels of myosin II cortical enrichment. In these cell lines, a slight increase followed by a measurable decrease in the levels of myosin II was observed, even though the timing was delayed and the signal was not as robust. However, 3XALA, GbpC null, and GskA null cell lines exhibited defects in myosin II filament recruitment to the cell cortex. While 3XALA cells showed difficulties in myosin II filament disassembly, GbpC null and GskA null cells did not seem to respond to cAMP stimulation significantly.

Analysis of MHCK D Knockout Electroporation via PCR

To determine proper uptake of the MHCKD-KO disruption cassette, PCR was performed on selected colonies. In order to make the disruption cassette, a blasticidin resistance gene was introduced into the *mhkD* gene (Figure 14) to construct the pBsr-Nsi-MHCKD-KO plasmid (Nagasaki, 2002) to be used to generate the *mhkA,B,C,D* quadruple knockout cell line. Therefore, with the proper set of primers, PCR was performed on each clone along with DNA gel electrophoresis to determine correct plasmid uptake. A total of 20 colonies were initially selected for PCR amplification. However, clone #14 became contaminated while waiting for plate confluency, and

therefore was eliminated from the remainder of the experiment. Figure 15 includes the PCR reactions from the MHCK D knockout (D-KO) plasmid and quadruple knockout clones #1 and #2 (#1 and #2). As shown in Figure 15, there is a PCR product present for both #1 and #2 approximately 1800 bases in length, while there is no band present with the D-KO plasmid. In addition, this same size PCR product band appears for all samples and Ax2 cells, while no PCR product appears with MHCK D- nor D-KO samples (Figure 15). This suggests that the *mhkD* gene was not disrupted through homologous recombination within my samples. Instead, the plasmid may have integrated nonspecifically in other parts of the genome through nonhomologous recombination leaving the *mhkD* gene intact. However, since none of the samples appeared to contain a disrupted form of this gene, the proposed microscopy studies could not be performed.

CHAPTER IV

DISCUSSION

In *Dictyostelium discoideum*, myosin II plays important roles in a variety of cellular processes. Cellular processes such as cell migration and chemotaxis are crucial for proper cell function, and therefore myosin II filaments are highly regulated through a series of controlled cellular signals. It is known that myosin heavy chain kinases (MHCKs) regulate myosin II. MHCKs phosphorylate three threonine residues on the heavy chain of myosin II, and allows the protein to dissociate from bipolar filaments to individual monomers localized to the cytoplasm of the cells. These monomers are incapable of driving contraction of actin filaments.

GbpC null cells exhibited no myosin II filament translocation when stimulated with cAMP. Therefore, it appears that GbpC is necessary for proper cAMP-mediated myosin II filament function. Prior research shows GbpC null cells exhibit defects in chemotaxis and myosin heavy chain phosphorylation (Bosgraaf et al., 2002). This, coupled with the results presented in this thesis, suggests that GbpC may regulate myosin II filaments at the MHCKs. Future research could examine the localization and activities of the MHCKs in cells lacking GbpC to determine if this is the mechanism by which GbpC functions. Also mentioned previously, GskA null cells also display defects in myosin II filament assembly. When GskA null cells are stimulated with cAMP, there is no visible change in myosin II filament positions about the cell.

In this thesis work, myosin II filaments seem to be able to assemble at the cell cortex of PkbA null cells. However, the cell is unable to complete the process properly, since after cAMP stimulation, the signal of cortical myosin II in PkbA null cells is not as robust as wild type Ax2 cells. Therefore, PkbA is needed for proper myosin II filament regulation, though not essential for the process itself. Previous literature suggests that PkbA may have a role in myosin II filament turnover due to its potential to co-localize with myosin II to the cell cortex (Meili et al., 1999). Additionally, when Meili disrupted the *pkbA* gene, the cells were unable to aggregate normally. Together, this may imply that PkbA indeed regulates myosin II filament turnover in some capacity. In order to understand how this occurs, future mutagenesis studies would need to be performed.

Along with PkbA null cells, RegA null cells also seemed to assemble minimally in response to cAMP stimulation. RegA is a phosphodiesterase, normally responsible for removing a phosphate from cAMP, establishing a concentration gradient and allowing *Dictyostelium* cells to migrate in a given direction. In RegA null cells, we observed a slight increase in myosin II at the cell cortex, once cells were given cAMP. This response may be due to the lack of the phosphodiesterase, creating a steady level of cAMP and inhibiting the concentration gradient the cells need to remodel myosin II. Prior research revealed that RegA null cells form more lateral pseudopodia, indicating defects in the regulation of myosin II filaments (Wessels et al., 2000). Future research of RegA could uncover what proteins it directly affects that ultimately drive myosin II filament assembly or disassembly.

Finally, VwkA null cells exhibit an intriguing phenotype when stimulated with cAMP. Myosin II filaments initially dissociate from the cell cortex before being recruited to the cell cortex. VwkA, a member of the *Dictyostelium* alpha kinase family, does not act in the same manner as the MHCKs. However, previous research suggests that VwkA may regulate myosin II filaments directly in some capacity. One study revealed that VwkA null cells were unable to complete cytokinesis when grown in suspension culture, however the phenotype was recovered when cells were grown in Petri dishes (Betapudi et al., 2005). This indicates that when VwkA cells are in suspension culture, either myosin II filaments or one of the upstream regulators are unable to function without VwkA. Future research between VwkA and cAMP-stimulated myosin II translocation could focus on which of the upstream regulators (e.g. MHCKs or MHC phosphatases) are affected by the lack of VwkA.

By way of the MHCK D knockout studies, we aspired to study the loss of all four MHCKs on the effect of myosin II filament assembly when cells were stimulated with cAMP. In order to achieve this, *mhkA/B/C null* cells were electroporated with a MHCK D disruption cassette, and allowed to incubate for homologous recombination. Previous research generated a *mhkD null* cell line using the same protocol and primers in this work (Egelhoff et al., 2006). However, based on the PCR results, none of the isolated colonies contained the disrupted version of MHCK D within the genomic DNA, so proposed research could not be completed with these cells. In future research, the efficacy of this project could be increased by obtaining a plasmid that has a separate antibiotic resistance marker to select for cells, instead of relying on diagnostic PCR. Also, it is possible that

the plasmid inserted itself into the genomic through nonhomologous recombination. Therefore, the PCR product would contain the intact and disrupted *mhkD* gene. However, with the selected primers, the disrupted version would not be visible. Future studies would need to include a method of excluding cells that were expressing the fully functional MHCK D, hopefully within cells that already lack MHCK A/B/C expression.

This thesis work intended to discover regulators of myosin II filament assembly within the context of cAMP stimulation. Myosin II filament assembly is a highly coordinated process due to its involvement in a variety of cellular processes such as cell migration, cytokinesis, and cytoskeletal remodeling. By understanding how myosin II is regulated in these contexts, we gain insight in correcting issues when processes are defective. Future studies of myosin II regulation can potentially uncover how each of the above upstream regulators plays into the regulation of filament assembly, and ultimately reveal the full pathway of cAMP-mediated myosin II filament translocation.

REFERENCES

Betapudi V, Mason C, Licate L, Egelhoff TT. Identification and Characterization of a Novel α-Kinase with a von Willebrand Factor A-like Motif Localized to the Contractile Vacuole and Golgi Complex in *Dictyostelium discoideum*. Devreotes P, ed. *Molecular Biology of the Cell* 2005;16(5):2248-2262.

Betapudi, V., Shoebotham, K., & Egelhoff, T. T. (January 01, 2004). Research Reports - Generation of double gene disruptions in *Dictyostelium discoideum* using a single antibiotic marker selection. *Biotechniques*, 36, 1, 106.

Bosgraaf, L., Russcher, H., Smith, J. L., Wessels, D., Soll, D. R., & Van, H. P. J. (January 01, 2002). A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium*. *The EMBO Journal*, 21, 17, 4560-70.

Bosgraaf, L., & van Haastert, P. J. (January 01, 2006). The regulation of myosin II in *Dictyostelium*. *European Journal of Cell Biology*, 85, 9-10.

Egelhoff, T. T., Lee, R. J., & Spudich, J. A. (January 01, 1993). *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo. *Cell*, 75, 2.)

Goldberg, J., Wolpin, E., Bosgraaf, L., Clarkson, B., Haastert, P., & Smith, J. (2006). Myosin light chain kinase A is activated by cGMP-dependent and cGMP-independent pathways. *FEBS Letters*, 2059-2064.

Kessin, R.H. 2001. *Dictyostelium*: evolution, cell biology, and the development of multicellularity. Cambridge University Press, Cambridge, UK. 294 pp.

Kolman, M.F., L.M. Futey, and T.T. Egelhoff. 1996. *Dictyostelium* myosin heavy chain kinase A regulates myosin localization during growth and development. *J Cell Biol*. 132:101-9.

Manstein, D. J., Titus, M. A., De, L. A., & Spudich, J. A. (January 01, 1989). Gene replacement in *Dictyostelium*: generation of myosin null mutants. *The EMBO Journal*, 8, 3, 923-32.

Meili, R., Ellsworth, C., Lee, S., Reddy, T. B., Ma, H., & Firtel, R. A. (January 01, 1999). Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in *Dictyostelium*. *The EMBO Journal*, 18, 8, 2092-105.

Nagasaki, A., Itoh, G., Yumura, S., & Uyeda, T. Q. P. (January 01, 2002). Novel Myosin Heavy Chain Kinase Involved in Disassembly of Myosin II Filaments and Efficient Cleavage in Mitotic *Dictyostelium* Cells. *Molecular Biology of the Cell*, 13, 12, 4333.

Rico, M., & Egelhoff, T. T. (January 01, 2003). Myosin heavy chain kinase B participates in the regulation of myosin assembly into the cytoskeleton. *Journal of Cellular Biochemistry*, 88, 3, 521-32.

Sasaki, N., R. Ohkura, and K. Sutoh. 2002. *Dictyostelium* myosin II as a model to study the actin-myosin interactions during force generation. *J Muscle Res Cell Motil*. 23:697-702.

Schilde, C. (2004). GSK3 is a multifunctional regulator of *Dictyostelium development*. *Development*, 4555-4565. Retrieved May 28, 2015, from http://dev.biologists.org/content/131/18/4555.long

Stites, J., Wessels, D., Uhl, A., Egelhoff, T., Shutt, D. and Soll, D. R. (1998), Phosphorylation of the *Dictyostelium* myosin II heavy chain is necessary for maintaining cellular polarity and suppressing turning during chemotaxis. *Cell Motil. Cytoskeleton*, 39: 31–51

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol*. 28:9-29.

Teo, R., Ryves, W. J., Reddy, J. V., Rogers, B. J., Harwood, A. J., Lewis, K. J., & Forde, J. E. (August 01, 2010). Glycogen synthase kinase-3 is required for efficient *Dictyostelium* chemotaxis. *Molecular Biology of the Cell*, 21, 15, 2788-2796.

Thomason, P. (1998). An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*. *The EMBO Journal*, 2838-2845. Retrieved May 28, 2015, from http://emboj.embopress.org/content/17/10/2838.long

Vaillancourt, J. P., Lyons, C., & Côté, G. P. (January 01, 1988). Identification of two phosphorylated threonines in the tail region of *Dictyostelium* myosin II. The *Journal of Biological Chemistry*, 263, 21, 10082-7.

Weber, I., Neujahr, R., Du, A., Köhler, J., Faix, J., & Gerisch, G. (May 01, 2000). Two-step positioning of a cleavage furrow by cortexillin and myosin II. *Current Biology*, 10, 9, 501-506.

Wessels, D. J., Zhang, H., Reynolds, J., Daniels, K., Heid, P., Lu, S., Kuspa, A., ... Soll, D. R. (January 01, 2000). The internal phosphodiesterase RegA is essential for the suppression of lateral pseudopods during *Dictyostelium* chemotaxis. *Molecular Biology of the Cell*, 11, 8, 2803-20.

Xu, X. S., Lee, E., Chen, T., Kuczmarski, E., Chisholm, R. L., & Knecht, D. A. (January 01, 2001). During Multicellular Migration, Myosin II Serves a Structural Role Independent of its Motor Function. *Developmental Biology*, 232, 1, 255-264.

Yumura, S., Yoshida, M., Betapudi, V., Licate, L. S., Iwadate, Y., Nagasaki, A., Uyeda, T. Q.-P., ... Egelhoff, T. T. (January 01, 2005). Multiple Myosin II Heavy Chain Kinases: Roles in Filament Assembly Control and Proper Cytokinesis in *Dictyostelium*. *Molecular Biology of the Cell*, 16, 9, 4256.

Zang, J.-H., Cavet, G., Sabry, J. H., Wagner, P., Moores, S. L., & Spudich, J. A. (January 01, 1997). On the Role of Myosin-II in Cytokinesis: Division of *Dictyostelium* Cells under Adhesive and Nonadhesive Conditions. *Molecular Biology of the Cell*, 8, 12, 2617.

APPENDIX A

TABLES

Table 1. List of Cell Lines Used in Aim 1.

LIST OF KO CELL LINES		
Cell Line	**Gene Disrupted**	**Phenotype**
HS1-3XALA	Threonine residues changed to alanines	Overassembly of myosin II in cytoskeleton;
GskA-bsr	*gskA*	Poor chemotaxis toward cAMP; aggregation delays
GbpC-bsr	*gbpC*	Delays in aggregation
MHCK A-B-C-	*mhkA, mhkB, mhkC*	Myosin overassembly; Defects in cytokinesis and chemotaxis
PkbA-bsr	*pkbA*	Delays in aggregation
RegA-bsr	*regA*	Increased lateral pseudopod formation
VwkA-bsr	*vwkA*	Increased myosin II assembly; delayed development; defects in chemotaxis

The above table lists all experimental cell lines used in this thesis work, including noted phenotypic differences as compared to wild type Ax2 *Dictyostelium* cells.

Table 2. Source Information on Experimental Cell Lines.

DATABASE INFORMATION FOR EXPERIMENTAL CELL LINES		
Cell Line	**Strain ID**	**Reference ID**
HS1-3XALA	DBS0236550	Egelhoff et al., 1993
GskA-bsr	DBS0236114	Schilde et al., 2004
GbpC-bsr	DBS0235994	Goldberg et al., 2006
MHCK $A^-B^-C^-$	DBS0235649	Yumura et al., 2005
PkbA-bsr	DBS0236784	Meili et al., 1999
RegA-bsr	DBS0236257	Thomason et al., 1998
VwkA-bsr	DBS0237046	Betapudi et al., 2005

Strain ID refers to the catalog number on Dictybase.org where the cell lines were ordered. Reference ID refers to the paper in which the cell line was first mentioned.

APPENDIX B

FIGURES

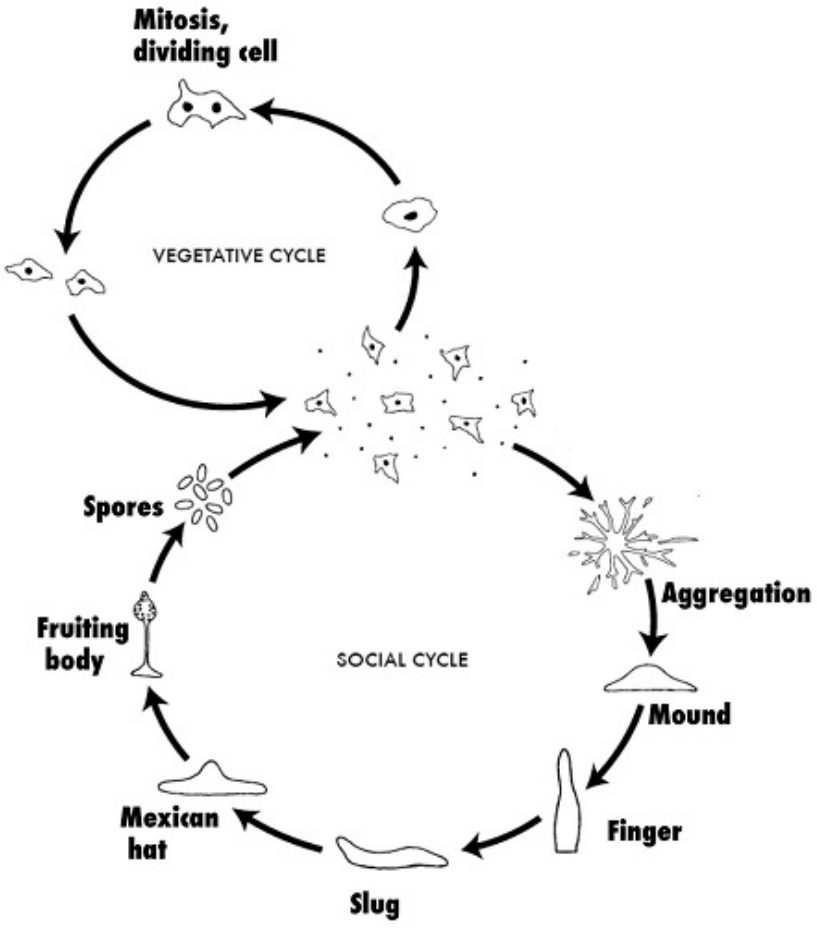

Figure 1. Developmental Stages of *Dictyostelium discoideum.* When nutrients are plentiful, *D. discoideum* exists in a unicellular state feeding on bacteria. Upon starvation, the cells will aggregate and progress through a series of stages, ultimately forming spores that can be released to grow in more fertile areas (Brown and Strassmann).

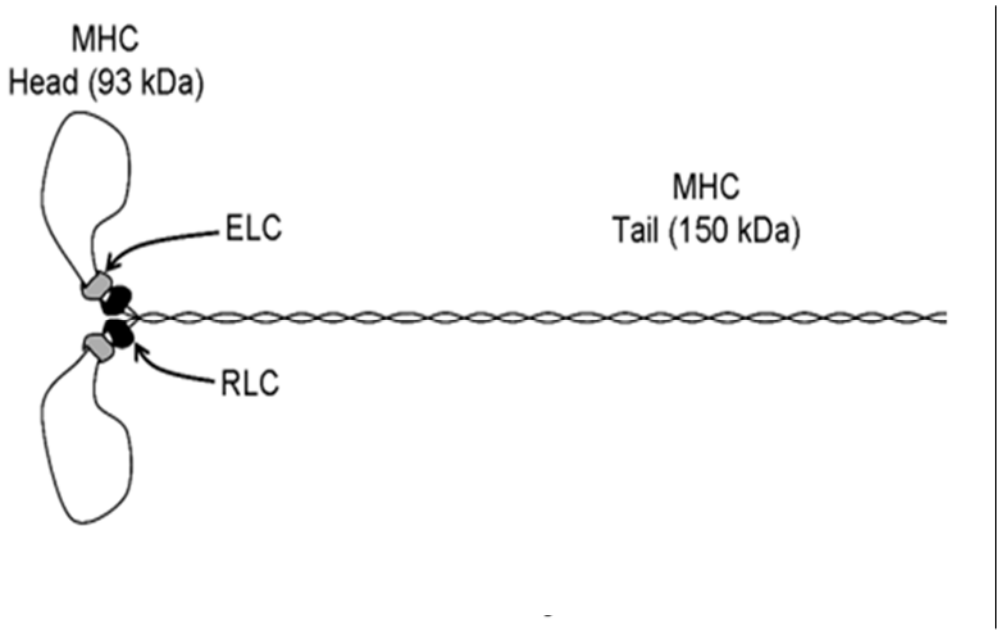

Figure 2. Schematic of a Myosin II Monomer. The myosin II monomer contains two globular head regions which are responsible for actin filament contraction. There are also two essential light chains and two regulatory light chains. The two heavy chain tails contain phosphorylation sites, which depending on whether they are phosphorylated or dephosphorylated, allows the myosin II monomers to disassemble or assemble, respectively.

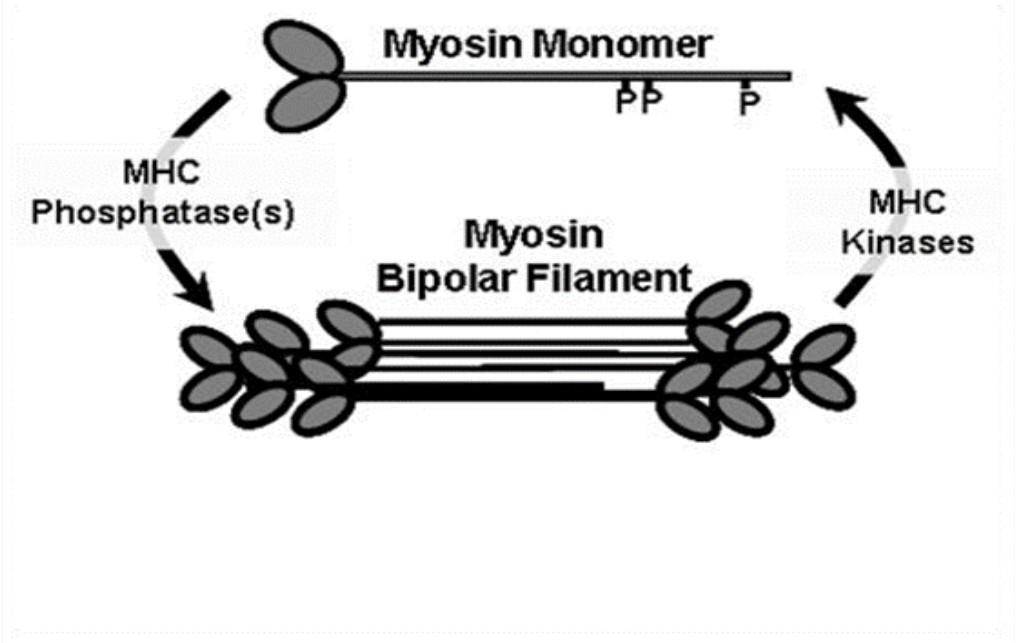

Figure 3. Regulation of Myosin II Assembly in *Dictyostelium*. Myosin II filament assembly is regulated by myosin heavy chain kinases (MHCKs) and myosin heavy chain phosphatases (MHCPs). Dephosphorylation of myosin II monomers allows them to assemble into active bipolar filaments, capable of contracting actin filaments. When MHCKs phosphorylate myosin II heavy chain tails, the bipolar filaments disassemble into inactive myosin monomers, incapable of contracting actin.

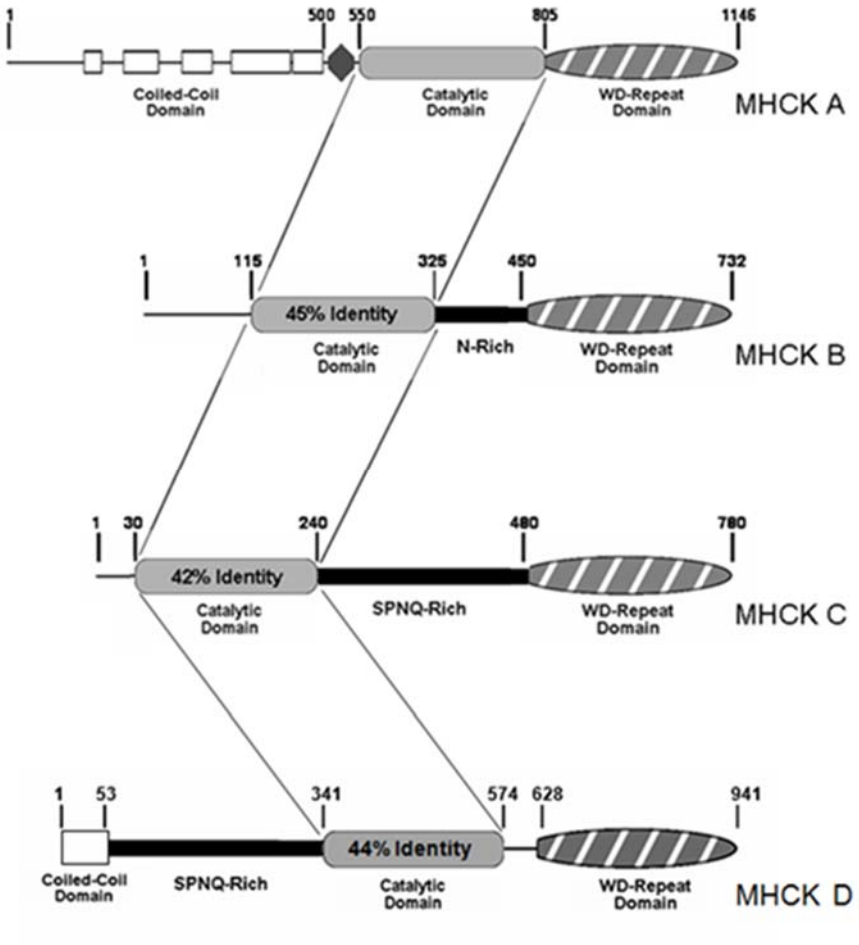

Figure 4. Schematic of the Domain Structure of the Myosin II Heavy Chain Kinase Family. The numbers represent amino acid positions within each kinase. The percent identity values given for the myosin heavy chain kinase (MHCK) B, C, and D catalytic domains is with respect to the sequence for MHCK A. The SNPQ-rich regions of MHCK C and D possess a high proportion of serines, asparagines, prolines, and glutamines. Likewise, the N-rich region of MHCK B is enriched in asparagine residues.

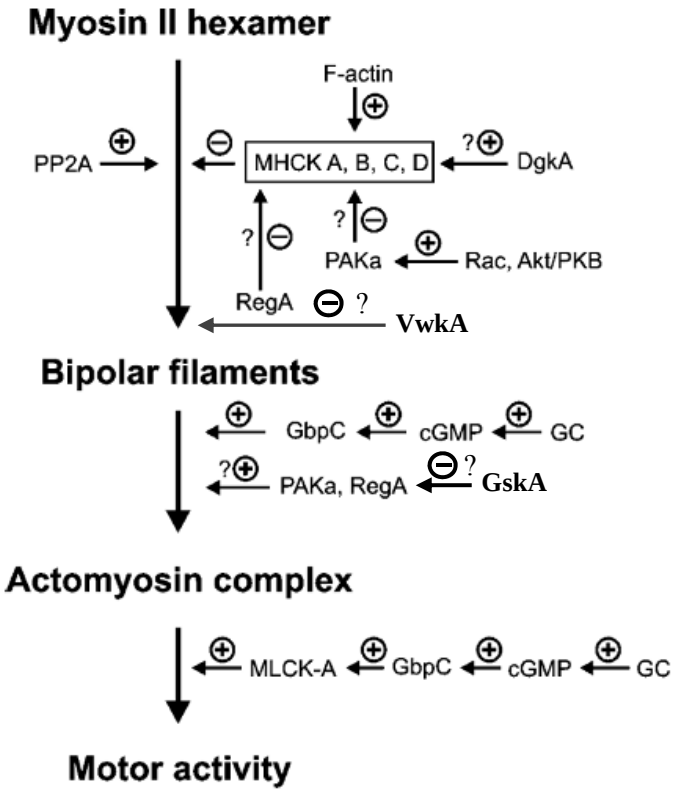

Figure 5. Diagram of Known and Putative Upstream Regulators of Myosin II. The upstream regulators chosen for this thesis research includes some known upstream regulators of myosin II. This image from Yumura et al. (2005) has been modified to include two other proteins that may affect myosin II filament regulation (VwkA and GskA). Three other upstream regulators (GpbC, RegA, and PkbA) are noted on the figure, and the various places of myosin II filament regulation they are thought to affect.

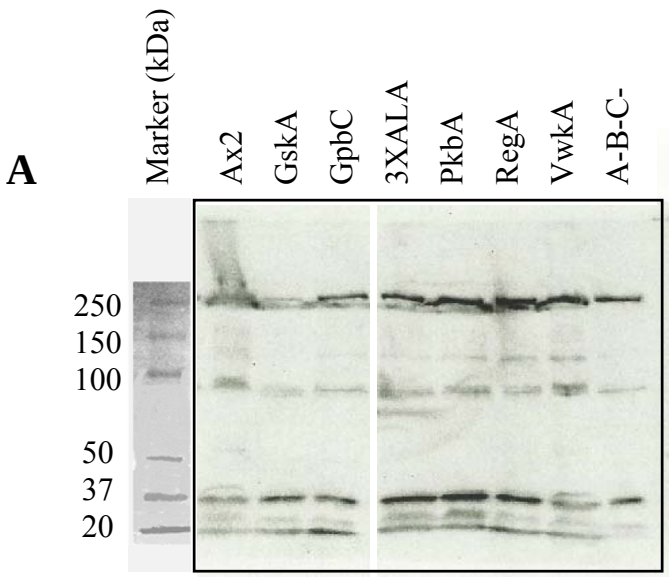

Figure 6A. Western Blot with Anti-GFP Antibody. Myosin II has a molecular weight of 243 kDa, while GFP has a molecular weight of approximately 25 kDa. The GFP-Myosin II (GFP-MYO) plasmid generates a product in which GFP is attached to the head region of the myosin II monomer, resulting in a combined molecular weight of approximately 270 kDa. Some breakdown product is noted at approximately 37 kDa, however, the bands around 270 kDa correspond to the same bands at 270 kDa in Figure 6B.

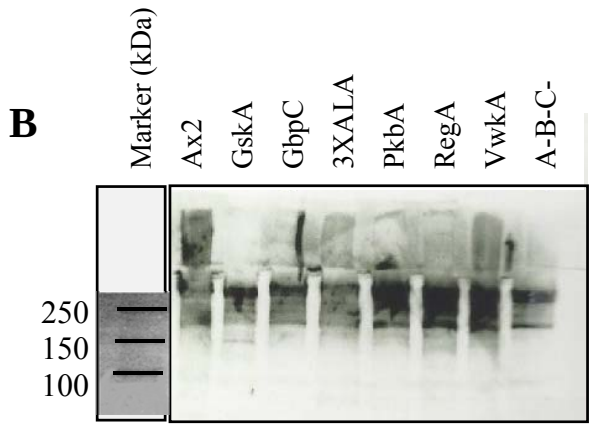

Figure 6B. Western Blot with Anti-Myosin II Antibody. Myosin II has a molecular weight of 243 kDa, while GFP has a molecular weight of approximately 25 kDa. The GFP-Myosin II (GFP-MYO) plasmid generates a product in which GFP is attached to the head region of the myosin II monomer, resulting in a combined molecular weight of approximately 270 kDa, corresponding to the same bands in Figure 6A.

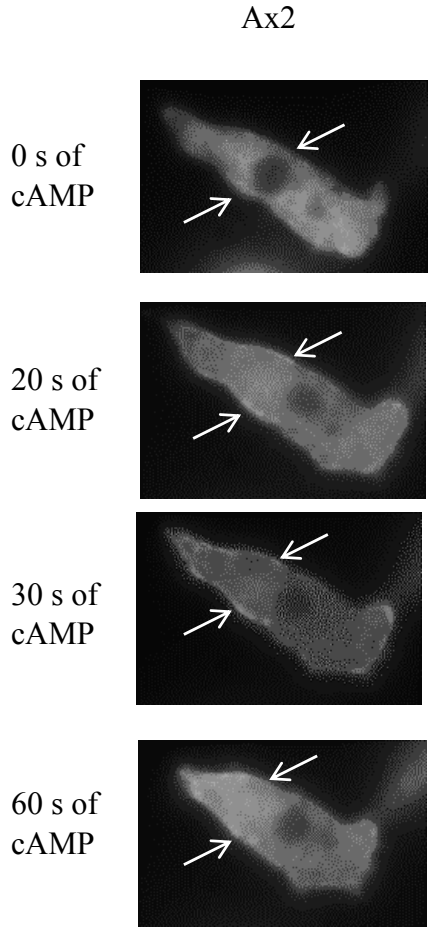

Figure 7. Myosin II Translocation Assay on Ax2 Cells. This image is representative of many cells exhibiting similar responses. Live cells photographed over time. When wild type Ax2 *Dictyostelium* cells are stimulated with cAMP, they will exhibit the characteristic increase in cortical myosin, followed by a decrease as myosin II disassembles and returns to the cytoplasm. This peak in cortical myosin usually occurs between 20-30 seconds post-cAMP addition. This timing and response was used as comparison for experimental cell lines. White arrows indicate regions where the intensity of GFP signal changes (increases or decreases) after the addition of cAMP.

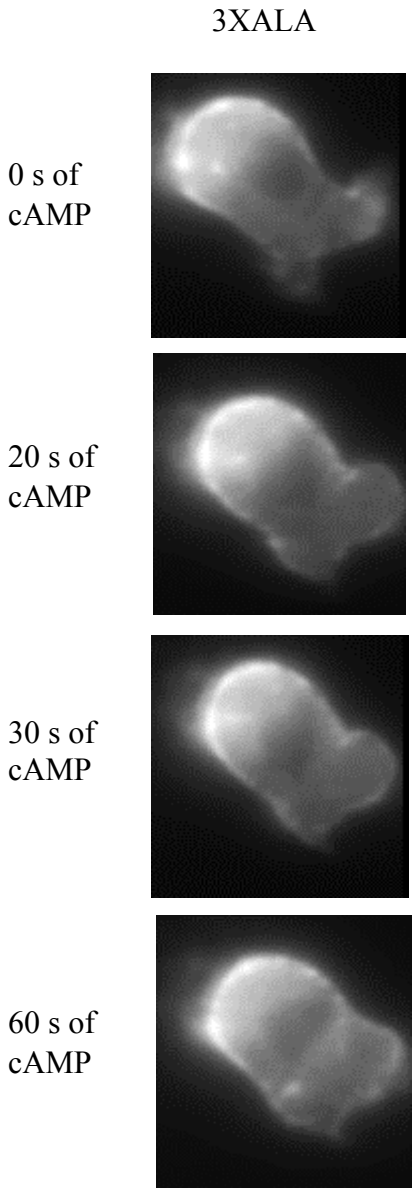

Figure 8. Myosin II Translocation Assay on 3XALA Cells. This image is representative of many cells exhibiting similar responses. Live cells photographed over time. When 3XALA cells are stimulated with cAMP, there is an increase in cortical enrichment, but fails to decrease. Because the myosin heavy chain tails cannot be phosphorylated in this mutant, the myosin II filaments cannot disassemble.

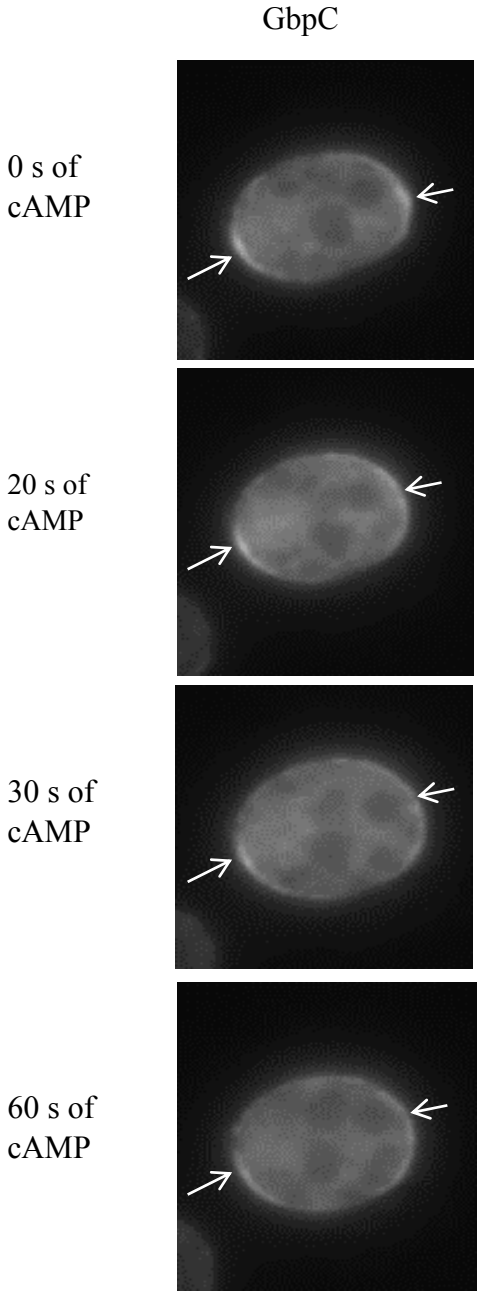

Figure 9. Myosin II Translocation Assay on GbpC Null Cells. This image is representative of many cells exhibiting similar responses. Live cells photographed over time. When GbpC null cells were stimulated with cAMP, there was no change in cortical myosin II levels, contrary to what is seen in Ax2 cells. Because cAMP-mediated myosin II translocation is not seen in cells lacking GbpC, this protein is essential to proper myosin II filament assembly. White arrows indicate regions where the intensity of GFP signal changes after the addition of cAMP.

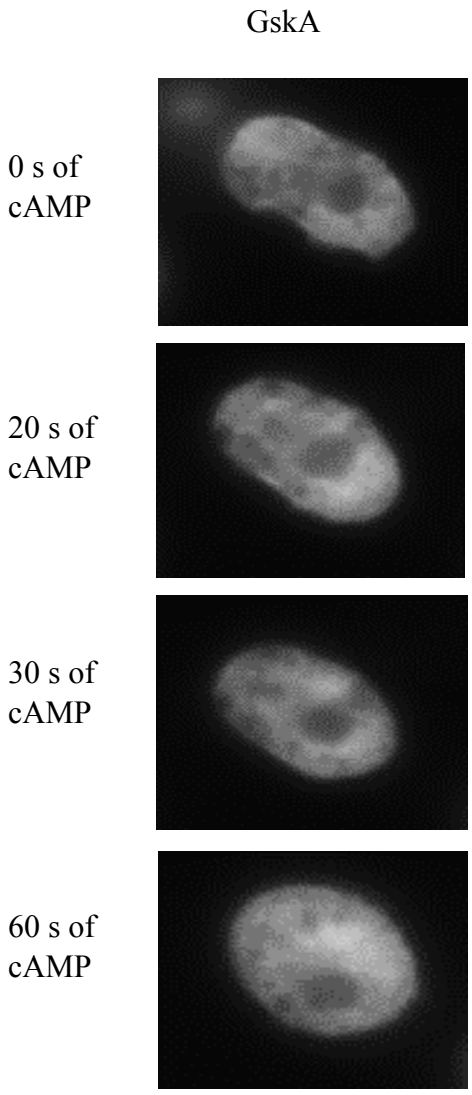

Figure 10. Myosin II Translocation Assay on GskA Null Cells. This image is representative of many cells exhibiting similar responses. Live cells were photographed over time. When stimulated with cAMP, GskA null cells do not exhibit cortical myosin II enrichment. Instead, the level of cortical and cytoplasmic myosin II remains relatively constant throughout the experiment. Therefore, GskA is required for proper cAMP-mediated myosin II filament regulation.

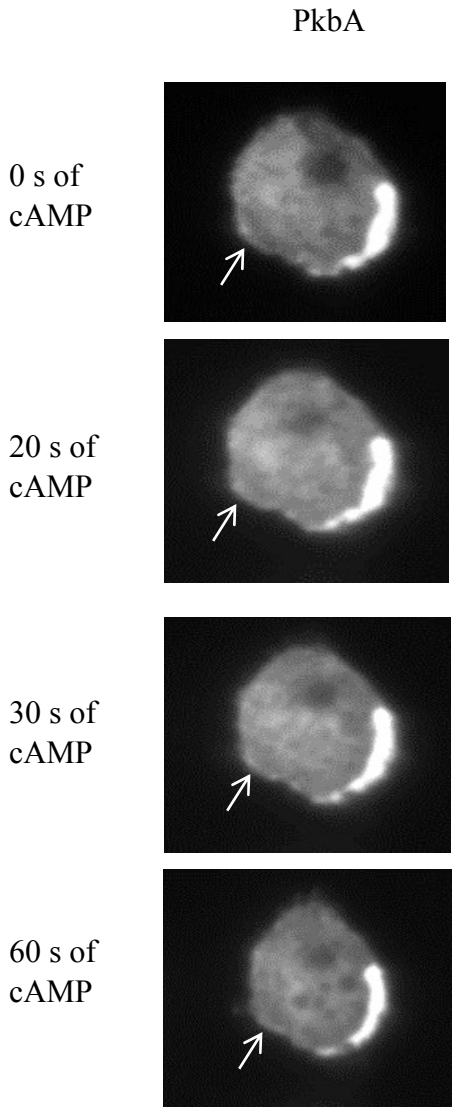

Figure 11. Myosin II Translocation Assay on PkbA Null Cells. This image is representative of many cells exhibiting similar responses. Live cells photographed over time. When stimulated with cAMP, PkbA null cells seem to exhibit defects in myosin II cortical enrichment. In addition, the cortex is enriched in localized areas. However, the phenotype is not abolished altogether, suggesting that PkbA is needed, in part, but its loss will not hinder myosin II filament regulation. White arrows indicate regions where the intensity of GFP signal changes after the addition of cAMP.

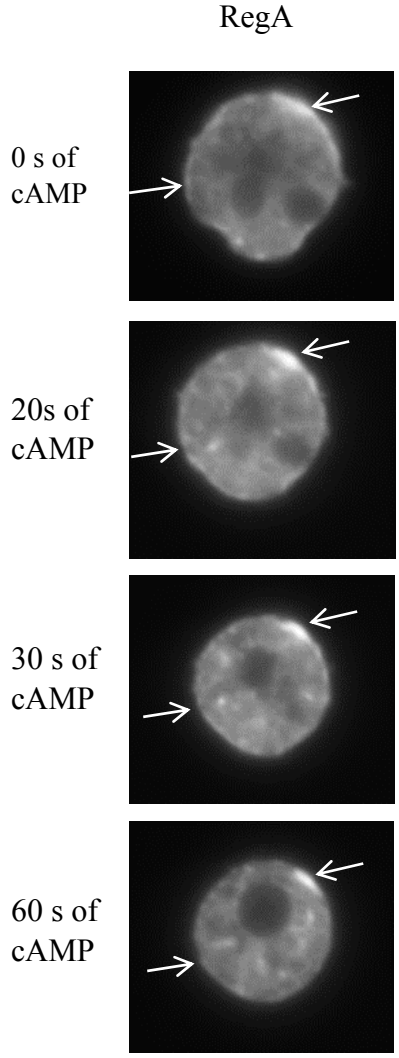

Figure 12. Myosin II Translocation Assay on RegA Null Cells. This image is representative of many cells exhibiting similar responses. Live cells photographed over time. When stimulated with cAMP, RegA null cells show reduced levels of enrichment. Also, myosin II is localized to certain areas around the cortex, as compared to Ax2 cells, which show enrichment around the entire cortex. Based on these images, RegA may be essential for proper regulation of myosin II filaments, but is not necessary for the process itself. White arrows indicate regions where the intensity of GFP signal changes after the addition of cAMP.

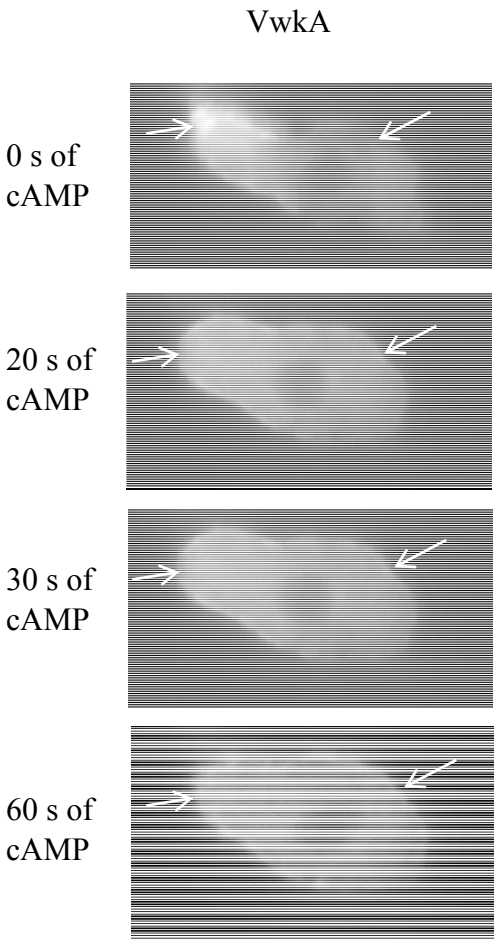

Figure 13. Myosin II Translocation Assay on VwkA Null Cells. This image is representative of many cells exhibiting similar responses. Live cells were photographed over time. Once stimulated with cAMP, VwkA null cells seem to show slight myosin II cortical enrichment as compared to Ax2 cells. However, once myosin II localizes to the cortex, it does not return to initial levels, indicating a slight defect in myosin II filament regulation. Thus, VwkA may be necessary for fully efficient regulation, but is not needed in this context. White arrows indicate regions where the intensity of GFP signal changes (increases or decreases) after the addition of cAMP.

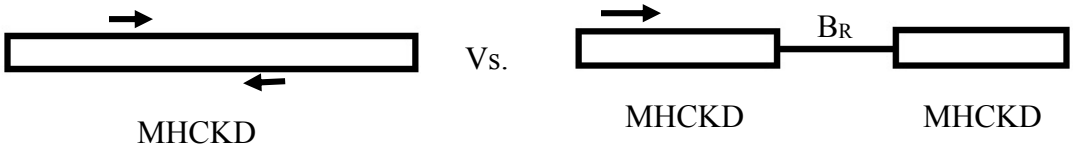

Figure 14. Strategy for Diagnostic PCR of *Dictyostelium* Clones in which the *mhkD* Gene Has Been Disrupted. The construct was created by inserting a blasticidin resistance gene (B_R) within the exon of the *mhkD* gene. With homologous recombination, the endogenous *mhkD* gene will be disrupted and a portion of the gene will be replaced with the gene for blasticidin resistance. Identification of cell lines in which homologous recombination has resulted in disruption of the mhkD gene will be achieved by PCR using forward and reserve primers that will anneal only to the intact (non-disrupted) *mhkD* gene. Thus, gDNA from clones in which the *mhkD* has not been disrupted will yield a PCR product. By contrast, the desired clones in which the *mhkD* gene has been disrupted will be identified based on the absence of a PCR product.

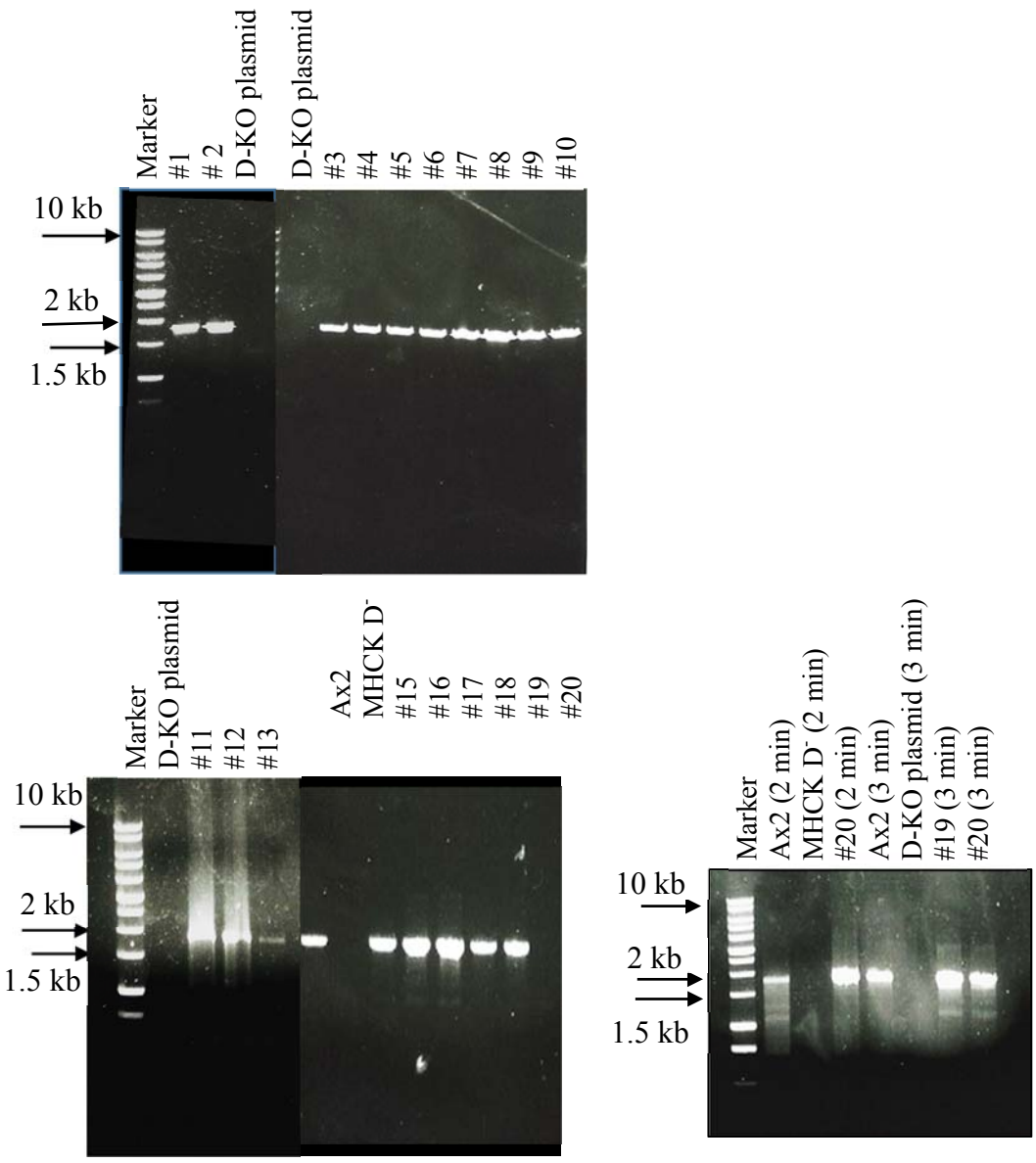

Figure 15. Analysis of PCR Reactions by Gel Electrophoresis. The left-most figures are the gels run at the initial cycles mentioned in the methods. At the initial cycle, all samples except for #20 had a PCR product at the same length of that isolated from wild type Ax2 cells. Neither the MHCKD-KO plasmid (D-KO plasmid), nor the MHCK D null cells (MHCK D^-) produced a product. The gel to the right indicate PCR products once the extension time was extended to 2 minutes and later, 3 minutes. The PCR product produced for #20 in each of these cases, resembled the length in Ax2 cells. Since neither the MHCKD-KO plasmid nor MHCK D null genomic DNA produced a PCR product with the given primers, samples with the disruption cassette should not have produced a PCR product. No such samples existed.

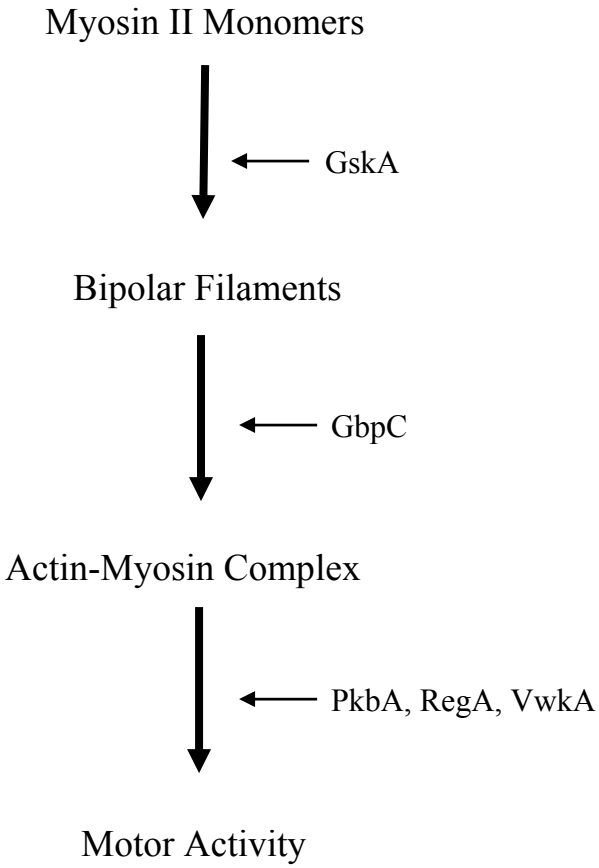

Figure 16. Updated Schematic of Myosin II Regulation. As a result of the work presented in this thesis, the above figure has been updated to include the effect of the studied upstream regulators. GskA null cells exhibited no change in myosin II filament function, indicating a defect in bipolar filament assembly. GbpC null cells did not exhibit a significant change in cortical myosin II, nor a shape change when stimulated, suggesting a role in establishing the actin-myosin complex. PkbA, RegA, and VwkA null cells were still able to complete myosin II filament translocation following cAMP stimulation, but exhibited defects in controlling cell shape. Therefore, these three may influence the motor activity of myosin II bipolar filaments.